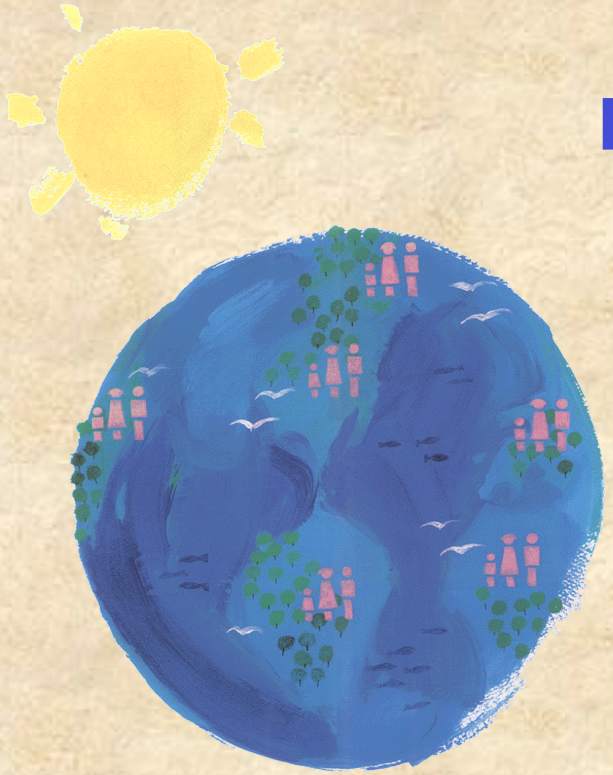
NICEATM

National Toxicology Program Interagency Center for the Evaluation Of Alternative Toxicological Methods

ICCVAM

Interagency Coordinating Committee on the Validation of Alternative Methods



Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) Test Method

BRD Summary

Expert Panel Meeting
January 11-12, 2005
Bethesda, Maryland

National Toxicology Program

NIEHS

Current U.S. Regulatory Status of HET-CAM

- ICCVAM agencies were surveyed and, to the best of their knowledge, HET-CAM test method data have not been submitted to U.S. Regulatory Agencies

Primary HET-CAM Data Sources

Study	Accuracy (Severes/Total)			Intralab (Severes/Total)		Interlab (Severes/Total)	
	GHS	EPA	EU	CVs	GHS classific.	CVs	GHS classific.
Gettings et al. (1991)	3/9	3/9	2/9	-	-	-	-
CEC (1991)	-	-	11/32	-	-	14	-
Gettings et al. (1994)	1/18	1/18	1/18	-	-	-	-
Bagley et al. (1992)	0/3	0/3	0/3	-	-	-	-
Vinardell and Macian (1994)	0/2	0/2	0/2	-	-	-	-
Balls et al. (1995) (Q)	15/45	10/45	14/48	-	-	40	15/29
Balls et al. (1995) (S)	11/17	6/12	11/19	-	-	8	11/5
Kojima et al. (1995)	3/5	3/5	3/5	-	-	-	-
Gettings et al. (1996)	8/23	10/25	6/25	-	-	-	-
Spielmann et al. (1996)	-	-	45/118	-	-	-	-
Hagino et al. (1999)	8/16	6/14	7/17	-	-	8	8/8

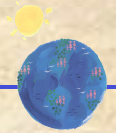
CV = coefficient of variation; classific. = classification

Other HET-CAM Reports Considered

- 39 other reports were identified that could not be used for an evaluation of accuracy or reliability due to the lack of:
  - comparative *in vivo* rabbit test data
  - quantitative *in vitro* data
- These reports discussed in Section 9
- To the extent possible, data requested from authors of studies considered most useful

HET-CAM Analysis Methods (1)

- Irritation Score (A) (IS(A))
  - Irritation responses are evaluated at 0.5, 2, 5 minutes
  - Time-dependent score are assigned to each endpoint
  - IS(A) is calculated by adding assigned scores
- IS(B)
  - Time of first appearance of endpoint is noted after application of test substance
  - IS(B) is calculated using empirically derived formula
- Q-Score
  - Calculated as ratio of test substance irritation score to investigator determined reference standard irritation score



HET-CAM Analysis Method (2)

- S-Score
  - Calculated as the highest total HET-CAM score for any endpoint evaluated
- IS and ITC
  - Two analysis methods used
  - Irritation score calculated as IS(A) or IS(B)
  - Threshold concentration defined as the lowest concentration required to produce a slight response after substance application

HET-CAM Database

- 246 different substances evaluated in 253 tests
- Distribution of substances among analysis methods
  - IS(A) = 64 substances (43 formulations, 21 chemicals)
  - IS(B) = 86 substances (52 formulations, 34 chemicals)
  - S-Score = 20 substances (all chemicals)
  - Q-Score = 49 substances (all chemicals)
  - IS and ITC = 118 substances (all chemicals or pharmaceutical intermediates)
- 20 Chemical classes tested*
  - Most frequent classes: alcohols, carboxylic acids, amines, and formulations
- 15 Product classes tested*
  - Most frequent classes: cosmetics, solvents, hair shampoos, soaps/surfactants

* Classes with at least 3 entries

Distribution of Tests Among Analysis Methods

Method	Number of Testing Laboratories						
	1	2	3	4	5	6	7
IS(A)	47	-	-	-	13	4	-
IS(B)	54	-	13	1	1	4	14
S-Score	2	7	6	5	-	-	-
Q-Score	2	6	1	40	-	-	-
IS and ITC	-	118	-	-	-	-	-

Major HET-CAM Protocol Variations

Study	# Eggs			Inc. Temp/ Humidity	Quantity Tested	Rinsing	Endpoints Evaluated**	Method of Analysis
	Neg	Treat	Pos					
Gettings et al. (1991)	-	-	-	-	-	-	H, VL, C	IS(B)
CEC (1991)	-	6	-	37.5/62.5%	0.3 mL or 0.1 g	20 secs after	H, L, C	IS(B)
Gettings et al. (1994)	-	3	-	38/60%	0.3 mL	-	H, L, C	IS(A) IS(B)
Gettings et al. (1996)	-	- 3	-	- 38/60%	0.1 mL 0.3 mL	-	D, H, C H, L, C	IS(A) IS(B)
Bagley et al. (1992)	2	4	-	37.5/62.5%	0.3 mL or 0.1 g	20 secs after	HY, H, C	IS(A)
Vinardell and Macian (1994)	2	6	2	-	0.3 mL	-	H, V, C	IS(B)
Balls et al. (1995)	-	-	-	-	-	3 mins after*	H, L, C	S-Score, Q-Score
Kojima et al. (1995)	-	4	-	37.6/~70%	0.2 mL	20 secs after	HY, H, C	IS(A)
Hagino et al. (1999)	-	4	-	37.6/~70%	0.2 mL or 0.2 g	20 secs after	HY, H, C	IS(A)
Spielmann et al. (1996)	-	3	-	-	-	5 mins after*	H, L, C	IS & ITC

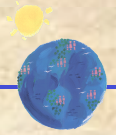
* For non-transparent substances only
** H = hemorrhage, VL = vascular lysis, C = coagulation, HY = hyperemia, L = lysis, D = dilation, V = vasoconstriction

Accuracy Analysis

- Ability to correctly identify ocular corrosives and severe irritants determined for
  - GHS classification system (Category 1)
  - EPA classification system (Category I)
  - EU classification system (R41)
- Accuracy statistics calculated:
  - for each HET-CAM test method protocol, by report and where appropriate
    - classifications were pooled into one classification per substance (i.e., majority call among studies used)
    - using individual studies, where a balanced design existed (multiple substances in multiple labs)

Analysis Method Accuracy - GHS

Analysis Method	Accuracy	Sensitivity	Specificity	False Positive Rate	False Negative Rate
IS(A)	75% (46/61)	67% (12/18)	79% (34/43)	21% (9/43)	33% (6/18)
IS(B)	85% (44/52)	100% (12/12)	80% (32/40)	20% (8/40)	0% (0/12)
Q-Score	62% (28/45)	100% (15/15)	43% (13/30)	57% (17/30)	0% (0/15)
S-Score	47% (8/17)	36% (4/11)	67% (4/6)	33% (2/6)	64% (7/11)



Recommended HET-CAM Version Accuracy

Statistic	GHS (n=52)	EPA (n=54)	EU (n=86)*
Accuracy	85% (44/52)	83% (45/54)	73% (63/86)
Sensitivity	100% (12/12)	93% (13/14)	95% (19/20)
Specificity	80% (32/40)	80% (32/40)	67% (44/66)
False Positive Rate	20% (8/40)	20% (8/40)	33% (22/66)
False Negative Rate	0% (0/12)	7% (1/14)	5% (1/20)

* Additional 32 chemicals available for EU analysis only (individual animal data not available for GHS or EPA classification)

HET-CAM GHS Accuracy By Chemical/Physical Class

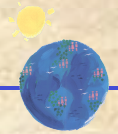
Class	# of Substances			False Positive Rate		False Negative Rate	
	Total	Cat 1	Cat 2A, 2B, NI	%	n	%	n
Overall	52	12	40	20	8/40	0	0/12
Formulation	50	12	38	18	7/38	0	0/12
- Hydroalcoholic formulation	9	3	6	33	2/6	0	0/3
- Oil/Water emulsion	18	1	17	24	4/17	0	0/1
- Surfactant-based formulation	23	8	15	7	1/15	0	0/8
Surfactant	2	0	2	50	1/2	-	-
Liquids	52	12	40	20	8/40	0	0/12

Additional HET-CAM Accuracy Analyses (EU)

Statistic	EU (n=86)	Spielmann et al. (1996) - IS10 (n=112)	Spielmann et al. (1996) - IS100 (n=108)
Accuracy	73% (63/86)	68% (76/112)	57% (62/108)
Sensitivity	95% (19/20)	80% (32/40)	88% (35/40)
Specificity	67% (44/66)	61% (44/72)	40% (27/68)
False Positive Rate	33% (22/66)	39% (28/72)	60% (41/68)
False Negative Rate	5% (1/20)	20% (8/40)	12% (5/40)

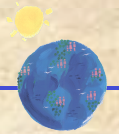
Limitations of HET-CAM IS(B) Accuracy

- Impact of differences in test method protocols between studies is unknown; limits conclusions
- Most substances evaluated using IS(B) analysis method were:
  - Nonsevere substances
  - Formulations
  - Tested as solutions or liquids
- Limited information on analysis method ability to accurately identify a variety of chemical classes, product classes, and physicochemical properties (i.e., solids)



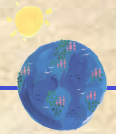
HET-CAM IS(B) Reliability Analysis

- **Intralaboratory Repeatability and Reproducibility**
  - Not conducted due to the lack of published intralaboratory HET-CAM data
- **Interlaboratory Reproducibility**
  - Qualitative analysis: Extent of agreement between testing laboratories when identifying corrosives and severe irritants
  - Quantitative analysis: Coefficient of variation (CV)



HET-CAM IS(B) Agreement Among Laboratories

% Interlaboratory Agreement	EU (3-5 labs, 32 substances)	
	%	n
100% (all)	47	15/32
≥60% (all)	91	29/32
100% (severes)	70	7/10
≥60%(severes)	100	10/10



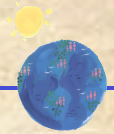
HET-CAM IS(B) Interlaboratory %CV Values

Coefficient of Variation Analysis	CEC (1991)
Mean (all substances)	34.1 (n=14)
Median (all substances)	33.1 (n=14)
Range (all substances)	6.6-74.9 (n=14)

*n = number of substances

Interlaboratory %CV values based on results from five laboratories

CV = Standard deviation/mean



Limitations of IS(B) Reliability

- Intralaboratory reliability unknown due to lack of published data
- Interlaboratory reproducibility based on a small number of substances (n=14)

Draft HET-CAM BRD Proposals

- A proposed version of HET-CAM, which evaluates development of hemorrhage, lysis, and coagulation of vessels on CAM
- A proposed standardized protocol
  - Proposed test method protocol follows the method provided by ZEBET with IS(B) analysis method
  - Decision criteria previously described by Kalweit et al. (1987)
  - Proposed test method protocol requires the use of positive and negative controls
- Proposed additional optimization studies, including:
  - Retrospective analysis of decision criteria used to identify corrosives and severe irritants
  - Evaluation of additional endpoints (e.g., trypan blue absorption) for potential inclusion in the calculation of irritancy potential
- Once optimized, additional validation studies to further characterize accuracy and reliability of the optimized test method version